Reference: *Biol. Bull.*, 146: 258–266. (April, 1974)

MOVEMENT OF PIGMENT GRANULES WITHIN MELANOPHORES OF AN ISOLATED FISH SCALE. EFFECTS OF CYTOCHALASIN B ON MELANOPHORES

TADAYUKI OHTA

Department of Biology, Aichi University of Education, Kariya City, Japan 448

Pigment granules within melanophores of various fish have been found to disperse in a physiological saline solution and to concentrate in M/7.5 (isotonic) KCl or adrenalin-physiological saline solutions. Such changes result from movement of the intracellular pigment granules (Spaeth, 1913; Matthews, 1931). There have been many reports by a considerable number of investigators on the intracellular movement of these granules, but the exact nature of the mechanism involved still lacks sufficient clarification (Ballowitz, 1914; Spaeth, 1913, 1916; Matthews, 1931; Biedermann, 1926; Marsland, 1944; Kamada and Kinosita, 1944; Kinosita, 1953, 1963; Bikle, Tilney and Porter, 1966; Wikswo and Novales, 1969, 1972; Ohta, 1971).

Lately, detailed electron microscopic studies on the fine structure of melanophores have been reported which cite the presence of many microtubules, microfilaments, endoplasmic reticulum and the like (Bikle, Tilney and Porter, 1966; Green, 1968). Wikswo and Novales (1969, 1972) have investigated the relationships between microtubules and movement of pigment granules, using colchicine for its so-called effect on intracellular microtubules (Borisy and Taylor, 1967a, 1967b; Shelanski and Taylor, 1967). The same authors indicate that colchicine inhibits concentration of the granules so as to foster their dispersion. Results of electron microscopy, moreover, revealed that microtubules in melanophores decrease significantly with colchicine treatment while microfilaments show relative increase. Malawista (1971), Novales and Novales (1972), McGuire and Moellmann (1972), and McGuire *et al.* (1972), have investigated cytochalasin B, known for its action upon microfilaments, for its effects upon the skin melanophore of the frog; however, there are no reports as yet on fish melanophore.

The present study aimed to investigate the relationships between various intracellular structures of melanophores and the movement of pigment granules. To achieve this, cytochalasin B was employed, and the effects on pigment granules movement of fish melanophore were investigated.

Material and Methods

The materials were melanophores from the isolated scale of *Oryzias latipes* (the wild type) which inhabit waters in and around the cities of Okazaki and Seto in Japan. Employing a pincette with sharp tips, the scale was carefully removed from the dorsoventral part of the fish and fixed, epidermis side down, to a glass coverslip mounted on a glass trough. Following isolation in this manner, the scale was kept immersed in a physiological saline solution, and after making certain that the granules had completely concentrated in an M/7.5 KCl solution, the test scale was again

put into the physiological saline solution for 10 min. Then, the melanophores were subjected to cytochalasin B (I. C. I., England).

For the physiological saline solution, an M/7.5 balanced solution according to Yamamoto (1939) was used. The cytochalasin B concentration was 1 μg/ml and 10 μg/ml-0.1% DMSO (dimethylsulfoxide)-physiological saline solution (pH adjusted to 7.2 by $NaHCO_3$). For the solution in which to concentrate the pigment granules, an M/7.5 KCl (pH 7.2 by $KHCO_3$) and 10^{-6} M adrenalin-physiological saline solution were employed. The experiments were run at room temperature, between 20–23° C.

Melanophore variations were measured photoelectrically (Fujii, 1959). Light was thrown on the photocell attached to one lens of the binocular microscope. Then, the photoelectric current was recorded (San'ei sokki, type 8C12-1119 test recorder) after amplification by an amplifier (San'ei sokki, type 1205C). Microscopic observations of melanophores were performed with another lens.

Denervated melanophores were obtained by the operation performed upon fish scales as described in the previous paper (Ohta, 1972). The scale was first carefully isolated from the fish with a pincette under a stereoscopic microscope, immediately returned to the original position, and the fish was kept for over 18 hr at 28° C against a black container. Then the scale was removed once more and put in an M/7.5 (isotonic) KCl solution in which it exhibited no concentration whatsoever.

Results

Response of innervated melanophores to cytochalasin B

The innervated melanophores were subjected to cytochalasin B action at 1 μg/ml and 10 μg/ml concentrations, continuously for 60 min. The melanophores thus sustained a state of dispersion. With the above photoelectric method, a slight concentration response with recorded several minutes after drug action, but a constant state held thereafter. This was opposed to control (physiological saline solution) in which a constant state was maintained. Under microscopic observation, however, no concentration of pigment granules toward the centrosphere was found to be involved. Rather, there was movement out to the branch extremities so that pigment granules in the centrosphere were markedly reduced in number (Figs. 2A, 2B). This was the super-dispersion phenomenon, and with it was observed an extremely active Brownian movement of pigment granules. Almost no effect on melanophores was noticed from the 0.1% DMSO used to dissolve the cytochalasin B.

Next, after subjection to respective cytochalasin B concentrations for 30 and 60 min, concentration time of granules in M/7.5 (isotonic) KCl and then dispersing time in physiological saline solutions were observed. The results are indicated in Table I and II. At 30 min and the concentration induced by M/7.5 (isotonic) KCl solution thereafter, less time was required for pigment granules to attain full concentration as compared to control. However, this was entirely in terms of cytochalasin B concentration. There was little or no difference from controls after treatment with 0.1% DMSO. Five minutes following complete concentration in M/7.5 (isotonic) KCl solution and changing the external medium to a physiological saline one, the time for pigment granules to begin and complete dispersion was observed to be much shorter than in control (Table I, II).

TABLE I

Response of innervated melanophores to M/7.5 (isotonic) KCl and physiological saline solution after treatment with cytochalasin B for 30 minutes. Abbreviations used are; T-1: Length of time between exchange of external medium and start of pigment concentration (min); T-2: Length of time between exchange of external medium and complete pigment concentration (min); T-3: Length of time between exchange of external medium and start of pigment dispersion (min); T-4: Length of time between exchange of external medium and complete pigment dispersion (min).

	No. of tests	M/7.5 KCl		Physiological sol.	
		T-1	T-2	T-3	T-4
Control	11	0.9 ± 0.33	1.8 ± 0.33	3.0 ± 0.66	6.4 ± 1.47
1 μg/ml Cytochalasin B	11	0.6 ± 0.30	1.2 ± 0.39	1.5 ± 0.67	4.2 ± 0.98
10 μg/ml Cytochalasin B	10	0.2 ± 0.17	1.1 ± 0.27	0.5 ± 0.27	2.2 ± 0.49
0.1% DMSO	11	0.8 ± 0.30	1.7 ± 0.41	2.8 ± 0.60	5.9 ± 1.17

With 60-min exposure to the drug, there was more pronounced granule concentration or dispersion, and obviously both outstripped controls. The rate was much more accelerated than with 30-min exposure. Microscopic observation of pigment granule dispersion in the physiological saline solution showed that these granules tended to become very scarce in the centrosphere, the movement being out toward the branch extremities as dispersion occurred.

Response of denervated melanophores to cytochalasin B

Denervated melanophores were subjected to 60 min of 10 μg/ml cytochalasin B Results nearly paralleled those with the innervated melanophore. Both super-dispersion and an active Brownian movement were also observed.

Next, after 30- and 60-min exposure to 10 μg/ml cytochalasin B, the melanophores were subjected to 5 min of 10^{-6} M adrenalin followed by change of external medium to the physiological saline one. Then the time for pigment granules to con-

TABLE II

Response of innervated melanophores to M/7.5 (isotonic) KCl and physiological saline solution after treatment with cytochalasin B for 60 minutes; Abbreviations as in Table I

	No. of tests	M 7.5 KCl		Physiological sol.	
		T-1	T-2	T-3	T-4
Control	9	1.2 ± 0.43	2.1 ± 0.51	3.6 ± 0.61	8.3 ± 1.00
1 μg/ml Cytochalasin B	9	0.4 ± 0.24	1.3 ± 0.43	2.4 ± 0.92	5.7 ± 2.19
10 μg/ml Cytochalasin B	9	0.1 ± 0.08	0.9 ± 0.18	0.5 ± 0.27	1.8 ± 0.51
0.1% DMSO	9	1.3 ± 0.56	2.2 ± 0.64	3.7 ± 0.96	7.5 ± 2.09

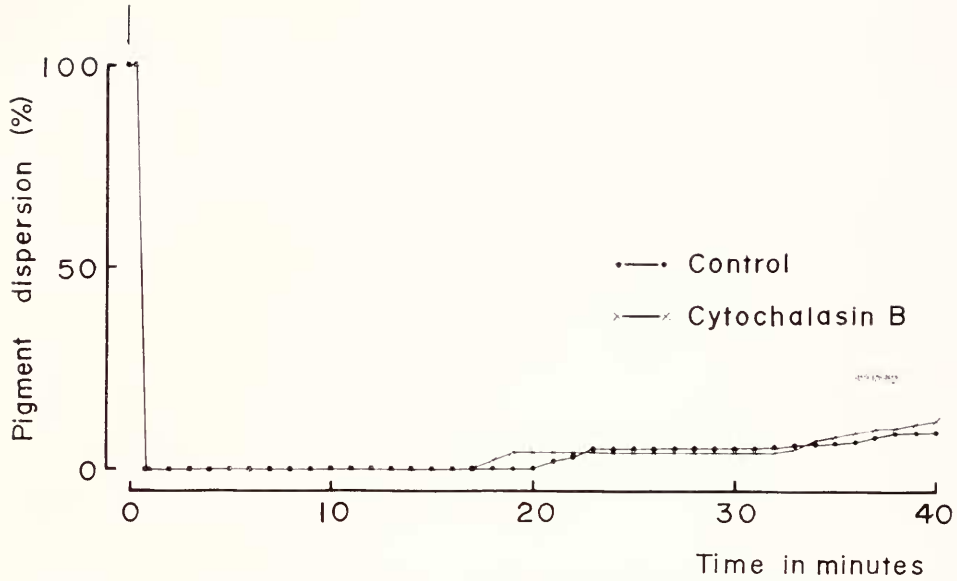

FIGURE 1. An example of cytochalasin B response of innervated melanophores concentrated with M/7.5 (isotonic) KCl solution. Horizontal indicates time after the solution was applied (arrow), room temperature, 21° C.

centrate and disperse was measured. Table III and IV provide the results. In the case of denervated melanophores exposed to adrenalin, the time until pigment granules began to concentrate and then reach complete concentration was extremely fast: at 30-min exposure, 0.1 min for the former, 0.9 min for the latter as against 0.6 and 2.3 min respectively with controls. Again, with dispersion, against the 17.4 min it took controls to attain 50% dispersion level, drug-treated pigment granules took 8.3 min, only half the time.

There was no special difference of time in concentration between 60-min or 30-min treatment, but in dispersion of pigment granules the dispersal process for 60 min was much faster than for 30-min exposure (Table III, IV).

TABLE III

Response of denervated melanophores to $10^{-6}M$ adrenalin and physiological saline solution after treatment with cytochalasin B for 30 minutes; abbreviations as in Table I except T-4: length of time between exchange of external medium and 50% pigment dispersion (min)

	No. of tests	Adrenalin		Physiological sol.	
		T-1	T-2	T-3	T-4
Control	10	0.6 ± 0.25	2.3 ± 0.83	5.9 ± 2.16	17.4 ± 3.50
10 μg/ml Cytochalasin B	10	0.1 ± 0.08	0.9 ± 0.09	2.0 ± 0.68	8.3 ± 1.60

TABLE IV

Response of denervated melanophores to 10^{-6} M adrenalin and physiological saline solution after treatment with cytochalasin B for 60 minutes; abbreviations as in Table I except T-4: length of time between exchange of external medium and 50% pigment dispersion (min)

	No. of tests	Adrenalin		Physiological sol.	
		T-1	T-2	T-3	T-4
Control	9	0.5 ± 0.14	2.2 ± 0.80	5.7 ± 2.71	16.3 ± 2.65
10 μg/ml Cytochalasin B	10	0.1 ± 0.12	1.0 ± 0.32	1.3 ± 0.98	4.3 ± 1.91

Cytochalasin B response of innervated melanophores concentrated with M/7.5 (isotonic) KCl solution

Cytochalasin B action at 10 μg ml was investigated with innervated melanophores fully concentrated in M/7.5 (isotonic) KCl solution and the results are given in Figures 1, 2C and 2D, and Table V. In M/7.5 (isotonic) KCl solution guanophores co-existing with the melanophores behave quite oppositely, but since their response is slow, photoelectric recording noted gradual dispersion often maximum concentration of pigment granules within melanophores had been obtained. However, once the guanophores were fully dispersed, an almost uniform response was recorded.

With cytochalasin B action, 3 of 10 samples showed dispersion degree greater than controls at 40 min (30%, 42%, 65%, respectively). At 10 or 20 min there was no great variation from controls, but at 30 or 40 min the mean dispersion rate nearly doubled. Nevertheless, this mean value difference was not statistically significant at 30 or 40 min.

Pigment granules dispersed very rapidly following exposure to cytochalasin B and change of external medium to a physiological saline one. In this situation nearly all pigment granules left the centrosphere, dispersing out to the branch extremities (Figs. 2E and 2F), a phenomenon often seen with diluted NaCl solution as reported earlier (Ohta, 1972).

TABLE V

Response (% of pigment granule dispersion at each time) of innervated melanophores concentrated with M/7.5 (isotonic) KCl solution to cytochalasin B

	No. of tests	After	After	After	After
		10 min	20 min	30 min	40 min
Control	10	1.6 ± 3.90	6.5 ± 6.66	9.5 ± 8.15	10.5 ± 9.92
10 μg/ml Cytochalasin B	10	0.8 ± 1.66	7.3 ± 9.63	15.0 ± 14.73	20.7 ± 19.64

Discussion

In 1967, Carter reported the interesting finding that, when cytochalasin B derived from mould metabolite separation acted upon fibroblasts, nuclear division remained normal but cell division was blocked. Subsequently, Schroeder (1969) found that, from an electron microscopic picture of the *Arbacia* egg treated with cytochalasin B, the contractile filaments which develop in the region of the cleavage furrow are notably absent, but no changes in the spindle-shaped microtubules appear. Follow-up studies have since been conducted not only on them but with Hela cells (Schroeder, 1970), lymphocytes (Ridler and Smith, 1968) and the like (Wessells *et al.*, 1971), and these have supported Carter's observations. Cytochalasin B is claimed to inhibit dispersion of pigment granules in frog skin melanophore (Malawista, 1971; Novales and Novales, 1972; McGuire and Moellmann, 1972; McGuire *et al.*, 1972). According to McGuire and Moellmann (1972) and McGuire *et al.* (1972), cytochalasin B even causes concentration of pigment granules though a dispersing agent is present, but does this while markedly reducing the number of microfilaments. Magun (1973), however, failed to find results as striking, so there would seem to be some disagreement about the validity of this particular effect of cytochalasin B.

On the other hand, electron microscopic observations have shown the presence of microtubules, microfilaments and endoplasmic reticulum in melanophores of fish (Bikle, *et al.*, 1966; Green 1968). Wikswo and Novales (1969, 1972), using colchicine, have researched its effect on melanophores of *Fundulus*. According to their physiological results, colchicine treatment of this type of melanophore leads to inhibition of pigment granule concentration and, conversely, promotion of dispersion. Upon electron microscopic observation, the microtubules within the melanophores were recognized to decrease and the microfilaments to increase. From these results, the same authors suggested that decrease of the microtubules may be related to inhibition of pigment granule concentration and the increase of the microfilaments to promotion of pigment granule dispersion.

Results of the present experiments indicate that treatment of melanophores with cytochalasin B, which has the earlier-mentioned effects, has a definite accelerative effect on movement—concentration and dispersion—of pigment granules, and that this effect is dependent upon time and drug concentration. This present observation, namely that cytochalasin B enhances the rate of response to adrenalin, is similar to that pointed out by McGuire and Moellmann (1972) and McGuire *et al.* (1972). Nevertheless, with melanophores in which pigment granules are in a state of dispersion, the use of cytochalasin B obtains no concentration at all. As evident from Figures 2A and 2B, the pigment granules are almost inexistent in the centrosphere, having dispersed to the branches. Lyerla and Novales (1972), in their observations and report on frog skin melanophore, have noted a similar effect of cytochalasin B as peculiar to the melanophore form. But with cytochalasin B, on the other hand, granules completely concentrated in M 7.5 (isotonic) KCl solution evidenced a somewhat scattered effect: after 30 or 40 min, dispersion levels greatly exceeded controls, but full dispersion was not attained.

Taken from the standpoint of cytochalasin B action, it is difficult to ascribe any direct kinetic capacity to the microfilaments when concentration or dispersion

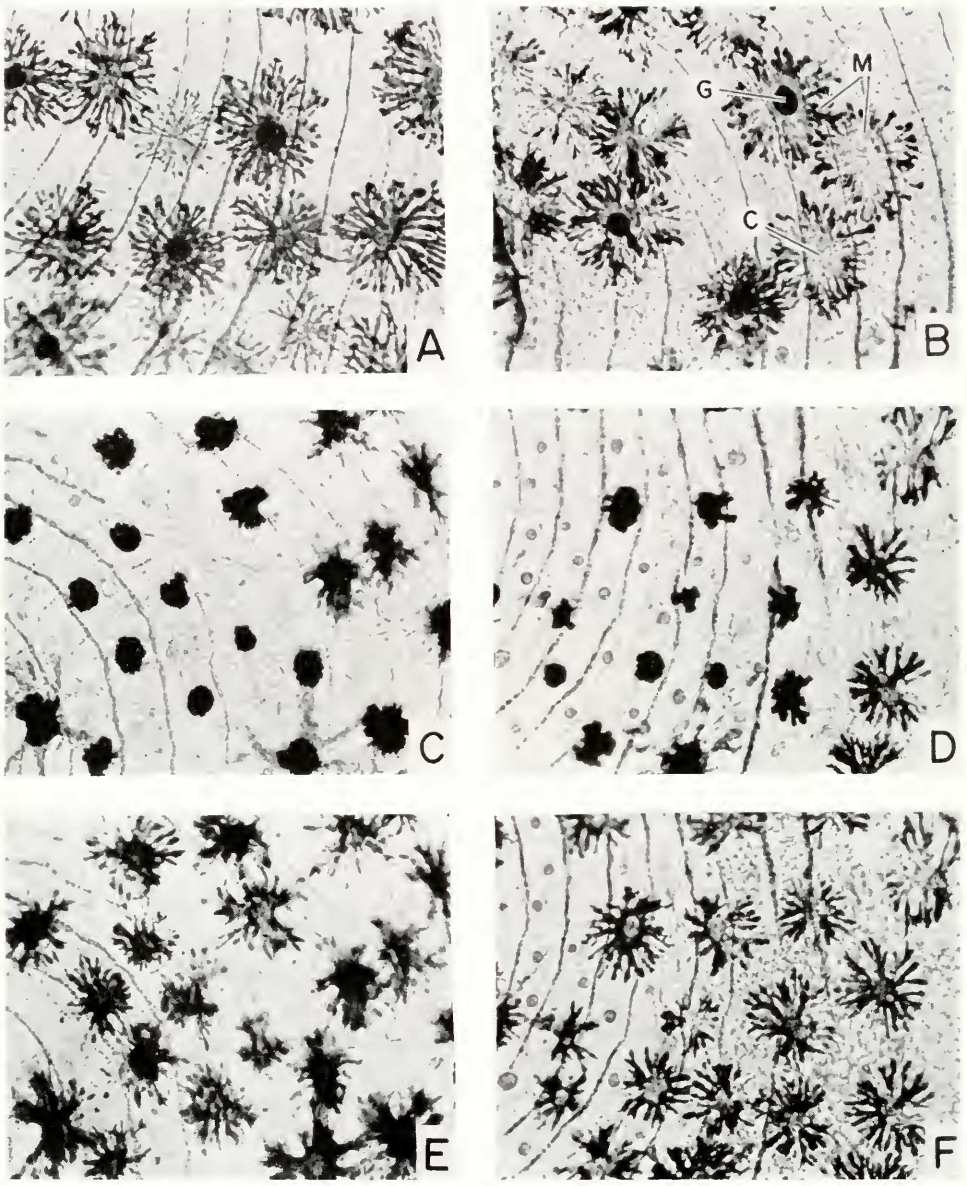

FIGURE 2. Photos of innervated melanophores treated with cytochalasin B; A, Control (in physiological saline solution; B, Innervated melanophore treated with 10 μg/ml cytochalasin B-physiol for 30 minutes; C, Control (in M/7.5 KCl solution); D, Innervated melanophore treated with 10 μg/ml cytochalasin B-M/7.5 KCl for 40 minutes; E, Control (dispersed condition of innervated melanophore in photo C by physiological saline solution); F, Dispersed condition of innervated melanophore in photo D by physiological saline solution; $\times$ 600. Symbols used are: M, Melanophore; G, Guanophore; C, Centrosphere.

movements take place with the granules inside melanophores. Instead, these pigment granules would appear to be inhibited in the course of normal movement by microfilaments. Further morphological observations by electron microscope are necessary, particularly upon microfilaments in the melanophore before and after use of cytochalasin B. Moreover, more detailed research from various aspects is required on the relations between pigment granule movement and elments of intracellular structure.

Summary

1. Employing melanophores from an isolated medaka (*Oryzias latipes*) scale, this study sought to clarify the mechanism of pigment granule movement by investigating cytochalasin B action.

2. Observations were made upon 1 μg/ml and 10 μg ml cytochalasin B innervated melanophores at 30 and 60 min, respectively, and followed by application of M/7.5 (isotonic) KCl, then physiological saline solution.

3. Results showed a marked acceleration of concentration and dispersion in pigment granules through cytochalasin B compared to control, and this effect was dependent upon time and drug concentration.

4. Approximately the same results obtained with denervated melanophores when adrenalin was employed instead of M/7.5(isotonic) KCl solution to concentrate pigment granules. The 0.1% DMSO (dimethysulfoxide) used to dissolve the cytochalasin B was not found to affect movement of pigment granules.

LITERATURE CITED

Ballowitz, E., 1914. Über die pigmentströmung in den farbstoffzellen und die kanälchenstruktur des chromatophorenprotoplasmas. *Arch. Ges. Physiol.*, **157**: 165–210.

Belanger, A. M., and R. C. Rustad, 1970. Movements of enchinochrome granules during the early development of *Arbacia* egg. *Biol. Bull.*, **139**: 415.

Biedermann, W., 1926. Vergleichende physiologie des integuments der wirbeltiere. *Erg. Biol.*, **1**(1): 1–342.

Bikle, D., L. G. Tilney and K. R. Porter, 1966. Microtunbules and pigment migration in the melanophores of *Fundulus heteroclitus* L. *Protoplasma*, **61**: 322–345.

Borisy, G. G., and E. W. Taylor, 1967a. The mechanism of action of colchicine. Binding of colchicine-^{3}H to cellular protein. *J. Cell Biol.*, **34**: 525–533.

Borisy, G. G., and E. W. Taylor, 1967b. The mechanism of action of colchicine. Colchicine binding to sea urchin eggs and the mitotic apparatus. *J. Cell Biol.*, **34**: 535–548.

Carter, S. B., 1967. Effects of cytochalasins on mammalian cells. *Nature*, **213**: 261–264.

Fujii, R., 1959. Mechanisms of ionic action in the melanophore system of fish 1. Melanophore concentrating action of potassium and some other ions. *Annot. Zool. Jap.*, **32**: 47–58.

Green, L., 1968. Mechanism of movements of granules in melanocytes of *Fundulus heteroclitus*. *Proc. Nat. Acad. Sci.*, **59**: 1179–1186.

Kamada, T., and H. Kinosita, 1944. Movement of granules in fish melanophore. *Proc. Imp. Acad., Tokyo*, **20**: 484–492.

Kinosita, H., 1953. Studies on the mechanism of pigment migration within fish melanophores with special reference to their electric potentials. *Annot. Zool. Jap.*, **26**: 115–127.

Kinosita, H., 1963. Electrophoretic theory of pigment migration within fish melanophore. *Ann. New York Acad. Sci.*, **100**: 992–1003.

Lyerla, T. A., and R. R. Novales, 1972. The effect of cyclic AMP and cytochalasin B on tissue cultured melanophores of *Xenopus laevis*. *J. Cell Physiol.*, **80**: 243–252.

McGuire, J., and G. Moellmann, 1972. Cytochalasin B: Effects on microfilaments and movemovent of melanin granules within melanocytes. *Science*, **175**: 642–644.

McGuire, J., G. Moellmann and F. McKeon, 1972. Cytochalasin B and pigment granule translocation. *J. Cell Biol.*, **52**: 754–758.

Magun, B., 1973. Two actions of cyclic AMP on melanosome movement in frog skin. *J. Cell Biol.*, **57**: 845–858.

Malawista, S. E., 1971. Cytochalasin B reversibly inhibits melanin granule movement in melanocytes. *Nature*, **234**: 354–355.

Marsland, D. A., 1944. Mechanism of pigment displacement in unicellular chromatophores. *Biol. Bull.*, **87**: 252–261.

Matthews, S. A., 1931. Observations on pigment migration within the fish melanophore. *J. Exp. Zool.*, **58**: 471–486.

Novales, R. R., and B. J. Novales, 1972. Effect of cytochalasin B on the response of chromatophores of isolated frog skin to MSH, theophylline, and dibutyryl cyclic AMP. *Gen. Comp. Endocrinol.*, **19**: 363–366.

Ohta, T., 1971. Movement of pigment granules within melanophores of an isolated fish scale. *Zool. Mag., Japan*, **80**: 453.

Ohta, T., 1972. Action of dilute solutions of NaCl and of KCl on melanophores in an isolated scale of *Oryzias latipes*. *Bull. Aichi Univ. Educ., Japan*, **21**: 87–96.

Ridler, M. A. C., and G. F. Smith, 1968. The response of human culture lymphocytes to cytochalasin B. *J. Cell Sci.*, **3**: 595–602.

Schroeder, T. E., 1969. The role of "contractile ring" filaments in dividing *Arbacia* egg. *Biol. Bull.*, **137**: 413–414.

Schroeder, T. E., 1970. The contractile ring. I. Fine structure of dividing mammalian (Hela) cells and the effects of cytochalasin B. *Z. Zellforsch.*, **109**: 443–449.

Shelanski, M. L., and E. W. Taylor, 1967. Isolation of a protein subunit from microtubules. *J. Cell. Biol.*, **34**: 549–554.

Spaeth, R. A., 1913. The mechanism of the contraction in the melanophores of fishes. *Anat. Anz.*, **40**: 520–524.

Spaeth, R. A., 1916. Evidence proving the melanophore to be a disguised type of smooth muscle cell. *J. Exp. Zool.*, **20**: 193–213.

Wessells, N. K., B. S. Spooner, J. F. Ash, M. O. Bradley, M. A. Ludueña, E. L. Taylor, J. T. Wrenn and K. M. Yamada, 1971. Microfilaments in cellular and developmental processes. *Science*, **171**: 135–143.

Wikswo, M. A., and R. R. Novales, 1969. The effect of colchicine on migration of pigment granules in the melanophores of *Fundulus heteroclitus*. *Biol. Bull.*, **137**: 228–237.

Wikswo, M. A., and R. R. Novales, 1972. Effect of colchicine on microtubules in the melanophores of *Funduls heteroclitus*. *J. Ultrastruct. Res.*, **41**: 189–201.

Yamamoto, T., 1939. Change of the cortical layer of the egg of *Oryzias latipes* at the time of fertilization. *Proc. Imp. Acad., Tokyo*, **15**: 269–271.